

N-Terminal Intramolecularly Conserved Histidines of Three Domains in *Gonyaulax* Luciferase Are Responsible for Loss of Activity in the Alkaline Region[†]

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ABSTRACT: *Gonyaulax* luciferase is a single-chain (~137 kDa) polypeptide comprising 111 N-terminal amino acids followed by three contiguous homologous domains (377 amino acids each). Each domain has luciferase activity, accounting for the earlier observation that proteolytic fragments (~35 kDa) of luciferase are active. The activity of the full-length native enzyme is maximal at pH 6.3, dropping to near zero at pH 8; the activity of fragments also peaks at pH 6.3 but remains high at 8. While the activity loss at higher pH might be thought to be associated with the conformation of the full-length protein, we show here that this is a property of individual domains. The three intramolecularly homologous domains, separately cloned and expressed in *Escherichia coli* as fusion proteins, exhibit pH–activity curves similar to that of the full-length enzyme. For each domain the removal of approximately 50 N-terminal amino acids resulted in an increase in the ratio of luciferase activity at pH 8 relative to that at pH 6.3, such that their pH–activity profiles mimicked that of the proteolytic fragments reported earlier. Replacement of N-terminal histidines by alanine by site-directed mutagenesis identified four that are involved in the loss of activity at high pH. This system illustrates an unusual, possibly unique mechanism for pH regulation of enzyme activity, which has been postulated to be responsible for the control of the characteristic flashes of bioluminescence.

All bioluminescent reactions, with the possible exception of fungal bioluminescence, involve the oxidation of a taxon-specific luciferin by molecular oxygen in an enzymatic reaction catalyzed by a specific luciferase (1, 2). Dinoflagellate luciferin is a novel tetrapyrrole (3), associated in the cell with a luciferin-binding protein (LBP)¹ (4). In *Gonyaulax polyedra* (*Lingulodinium polyedrum*), the species studied here, the luciferase is a single polypeptide chain (M_r ~137 000) containing three remarkably homologous domains, all having luciferase (LCF) activity as individual peptides (5).

In the cell, the three components (LCF, LBP, and luciferin) are located in scintillons, the bioluminescent organelles (6), and it has been postulated that a rapid and transient change in pH within scintillons is the biochemical trigger responsible for the characteristic flash of dinoflagellates. This was

suggested by the pH–activity profile of LCF, peaking at pH 6.3 and nil at pH 7.5 (7), and by the fact that acidification of scintillons isolated at pH 8 triggers flashing in vitro (8). The structure and location of scintillons are consistent with this hypothesis: the organelles project into the acidic vacuole and are enclosed by its membrane (6), along which the flash-triggering action potential is conducted (9).

In cellular extracts made at pH 7, luciferase activity was found in two molecular weight fractions, designated A (M_r ~140 000) and B (M_r ~35 000) luciferases (7, 10). The two fractions differed in their pH–activity profiles, the first exhibiting a maximal activity at pH 6.3, dropping down to zero at pH 8.0 (type A activity). The smaller B enzyme also exhibited a maximum at pH 6.3, but at pH 8.0 its activity remained high, about 50% of the maximum (type B activity). Extractions made at pH 8 yielded only full-length A-luciferase, whereas those performed at pH 6 gave exclusively the smaller size B-luciferase, and treatment of A with the protease subtilisin yielded B activity (10). These results suggested that the inhibition of activity in the alkaline region could be due to the conformation of the full-length molecule, which might be largely lost in peptides containing only an individual domain.

In the present work, the pH–activity profiles of peptides covering each of the three domains were compared to longer and full-length constructs, making use of the cloned *G. polyedra* luciferase gene and the glutathione S-transferase/luciferase (GST–LCF) fusion expression system (5). Contrary to expectation, all three individual full-length domains were found to have pH–activity profiles similar to that of

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¹ Abbreviations: LCF, luciferase; GST, glutathione S-transferase; PCR, polymerase chain reaction; LBP, luciferin binding protein; ORF, open reading frame.

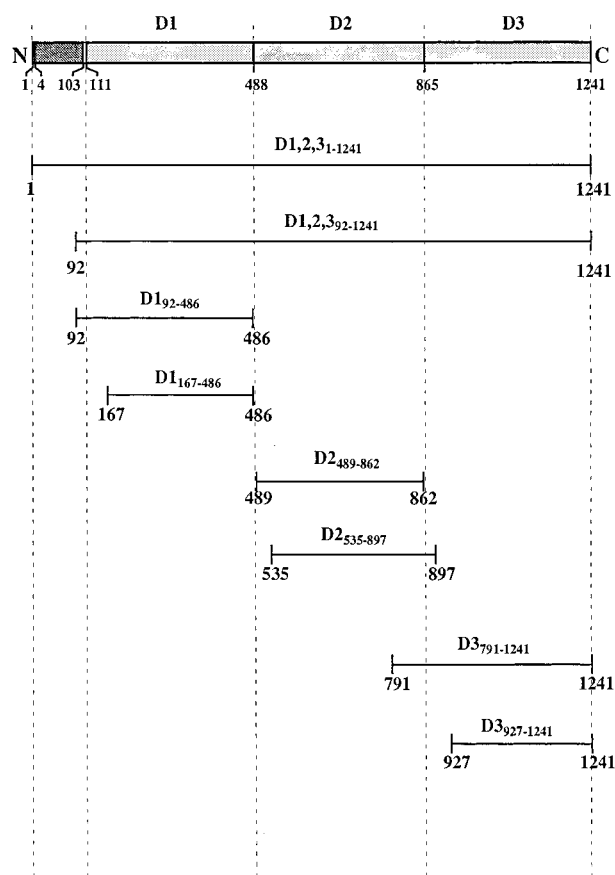


FIGURE 1: Schematic diagram showing the luciferase gene and its three homologous domains along with eight of the GST fusion proteins, two of which extend over all three domains. For each of the three domains there is one peptide encompassing all or most of its length and a second with about 50 amino acids absent from its N-terminus. Numbers indicate amino acid residues.

the full-length molecule. However, for all three domains, the removal of ~50 N-terminal residues resulted in substantial luciferase activity at alkaline pHs relative to that at pH 6.3. Intramolecularly conserved histidines within this region were replaced by alanine and shown to be responsible for the loss of activity at pH 8.

MATERIALS AND METHODS

Construction of GST-LCF Expression Plasmids. *lcf* cDNA fragments of different lengths corresponding to those shown in Figure 1 were generated by either polymerase chain reaction (PCR) amplification (D2₄₈₉₋₈₆₂) or restriction enzyme digestion of full-length *lcf* cDNA (all others). PCR was performed with a GeneAmp PCR System 9600 (Perkin-Elmer), with 35 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 60 °C, and 1.5 min of extension at 72 °C, followed by a final 10 min extension at 72 °C in a 100 μ L reaction mixture containing 1 \times buffer, 400 μ M dNTPs, 2 mM MgSO₄, and 1 unit of Vent polymerase (New England Biolabs), with the full-length *lcf* cDNA as template. The sequences of the forward and reverse primers used were 5'-TTGAGTACTTGCTGCGACCAGGGTTTCG-3' and 5'-GCAGAATTCAATGCCTTGGAAACCTT-3', respectively. Restriction enzyme digests of the full-length *lcf* cDNA were performed following the manufacturer's protocols. The *lcf* fragments generated were cloned in-frame with GST into the expression plasmid pGEX-3X (Pharmacia) and the

Table 1: Oligonucleotides Used for Site-Directed Mutagenesis^a

D2H35A.F	5'-CAAGAACGGACTGGCCGCGCCCAACTTC-3'
D2H35A.R	5'-GAAGTTGGGCGCGCCAGTCCGTTCTTG-3'
D2H45A.F	5'-GACGACGGCTTGGCCAAGCCCATGGAG-3'
D2H45A.R	5'-CTCCATGGGCTTGGCCAAGCCGTCGTC-3'
D2H60A.F	5'-CTCCACTGGGTTTGCTTACCTGCTGGAG-3'
D2H60A.R	5'-CTCCAGCAGGTAAGCAAACCCAGTGGAG-3'
D2H66A.F	5'-CTGCTGGAGGCCGCCGACCTTGGCGGC-3'
D2H66A.R	5'-GCCGCCAAGGTCGGCGGCCTCCAGCAG-3'

^a Numbering as in Figure 3.

resulting expression constructs were then transformed into *Escherichia coli* cells (JM109). The sequence of each *gst-lcf* expression plasmid was verified by restriction enzyme digestion and by DNA sequencing of the vector-insert junctions.

Expression of GST-LCF Fusion Proteins. GST-LCF fusion proteins were expressed as described previously (5). In brief, *E. coli* cells containing the *gst-lcf* expression plasmids were grown overnight at 23 °C in 2 \times YT-G medium (Pharmacia) supplemented with 100 μ g/mL of ampicillin, then diluted 10-fold in fresh medium and grown for 3 h at 23 °C. Isopropyl β -D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM and cells were grown for an additional 3 h at 23 °C. Cells were harvested by centrifugation for 5 min at ~3000g and resuspended on ice in 1 \times phosphate-buffered saline (Pharmacia; 50 μ L/mL of culture). A protease inhibitor [Pefabloc (Boehringer Mannheim) or phenylmethanesulfonyl fluoride (Sigma)] was added to a final concentration of 1 mM, and cells were sonicated three times for 10 s each at setting 5 of the sonicator (Misonix). Triton X-100 was added to a final concentration of 1% (v/v) and the resulting cell lysate solution was mixed for 30 min at 4 °C and then centrifuged at 12000g for 15 min at 4 °C. The supernatant was removed and stored at -80 °C until used. For measurements of specific activities (5), fusion proteins were purified by adsorption to and elution from glutathione-Sepharose 4B following the manufacturer's protocol (Pharmacia).

Site-Directed Mutagenesis. PCR-mediated mutagenesis of selected histidine residues in the N-terminal region of the second domain was performed with the QUICK-exchange kit (Stratagene). The PCR reaction was catalyzed by the high-fidelity DNA polymerase Pfu in the presence of the plasmid and pairs of forward and reverse mutagenic primers (Table 1). The full-length second domain (D2₄₈₉₋₈₆₂) cloned into pGEX-3X was used as the starting plasmid. To achieve substitutions at multiple sites, the next mutation was made starting with the mutated plasmid from the previous round. After the PCR reaction, the parental plasmid was selectively digested by the restriction enzyme *DpnI*, and the mixture was then used to transform XL1-Blue MRF' cells. For each transformation, 2-3 colonies were picked for plasmid isolation, and mutant plasmids were screened by DNA sequencing and subsequently reintroduced into *E. coli* ER2566 (NEB) for protein expression.

LCF Activity Assay. Droplets of GST-LCF (10-30 μ L) and a fixed volume of dinoflagellate luciferin (10 μ L) from *Pyrocystis lunula* (3) were placed at opposite sides of a vial in the photometer (11). The components were mixed by the rapid injection of 2 mL of 0.1 M phosphate buffer, thereby

initializing the reaction. Light emission was measured at 50 ms intervals over a course of at least 45 s/assay with a program written by Dr. Walter Taylor for LabVIEW (version 4.0), and the initial maximum intensity was taken as the measure of the luciferase activity. Assays of mutant luciferases were carried out in the same way.

For specific activity determinations, GST-LCF proteins were purified from two replicate *E. coli* cultures and a minimum of five assays were done for each preparation. The amount of luciferase used in each assay was determined by optical densitometry (NIH Image, version 1.6) of Coomassie blue-stained denaturing polyacrylamide gels with purified BSA as a standard. Molar concentrations of luciferase peptides were calculated on the basis of the molecular weight predicted from the nucleotide sequence of each clone. The large differences in specific activities were due to the different concentrations of the luciferin preparations used; all were well below saturation. Luciferase activity is linear with luciferin concentration over the range used.

For pH-activity profiles, cell-free extracts were prepared from at least two different cultures for each of the different GST-LCF constructs. Luciferase activity was measured in 0.1 M phosphate buffers ranging from pH 4.8 to 8.0 in increments of 0.4 pH unit. Five assays were done at each pH for each preparation, and activity is expressed relative to the maximum value measured for each replicate preparation.

RESULTS AND DISCUSSION

pH-Activity Profiles of Peptides Expressed in Vitro. The full-length luciferase cDNA (4037 bp) has an open reading frame (ORF) of 3723 bp and encodes the 136 994 Da luciferase molecule (5). The ORF (Figure 1) contains an N-terminal sequence of 111 amino acids, which is homologous to the N-terminal region of the substrate-binding protein, LBP. This is followed by three contiguous domains, designated D1, D2, and D3, which are homologous to one another. At the nucleotide level these are 75–80% identical in sequence overall and >95% identical in the more central regions, which presumably represent conserved catalytic domains.

The fact that the three repeat peptide domains each have luciferase activity (5) accounts for the activity of proteolyzed B-luciferase but not for its different pH-activity profile, since the present study demonstrates that single-domain peptides have A-type pH-activity profiles, similar to the full-length molecule. The active proteolytic fragments with type B activity are about 35 kDa, smaller than a full-length single domain, suggesting that activity at pH 8 might be due to peptides shorter than full domain length. We thus prepared and measured activities of truncated single-domain peptides as well as those spanning complete individual domains.

The eight clones illustrated in Figure 1 were expressed as GST fusion proteins in two separate runs and purified by affinity adsorption and elution. The results of luciferase assays at pH 6.3, given in Table 2 as specific activity on a molar basis, show that the full-length protein is the most active and that the removal of the 90 N-terminal residues resulted in the loss of one-half to one-third of the activity. The peptides covering complete individual domains had specific activities of 10–20% that of the full-length

Table 2: Luciferase Specific Activities at pH 6.3 of GST Fusion Proteins^a

peptide	run 1 specific activity	run 2 specific activity
D1,2,3 _{1–1241}	100 ± 16	100 ± 7.1
D1,2,3 _{92–1241}	67 ± 10	50 ± 5.1
D1 _{92–486}	11 ± 1	12.3 ± 2.3
D1 _{167–486}	3 ± 0.4	6.7 ± 0.7
D2 _{489–862}	10 ± 0.7	13.9 ± 1.1
D2 _{535–897}	5 ± 0.8	14.0 ± 1.3
D3 _{791–1241}	22 ± 3.6	14.3 ± 2.1
D3 _{927–1241}	4 ± 0.4	7.2 ± 0.8

^a Peptides (see Figure 1 for the individual identities) were expressed in *E. coli* in two separate runs, 1 and 2, purified, and measured for activity by mixing the constructs with luciferin at pH 6.3 and recording the initial maximum light intensity. Values are given in relative units, normalized to the value of the full-length luciferase in each run, with one unit corresponding to 2.8×10^9 quanta $s^{-1} \mu\text{mol}^{-1}$ in the first run and 3.6×10^9 quanta $s^{-1} \mu\text{mol}^{-1}$ in the second, the difference due to a difference in the concentration of the luciferin used in the assays. Differences in the specific activities for D2 in these measurements and those reported in Table 3 are similarly attributed to differences in luciferin concentration.

luciferase, consistent with the earlier conclusion that all three domains could be active concurrently in the full-length molecule (5). The removal of ~50 amino acids from each of the domains resulted in a further loss of activity at pH 6.3, with values ranging from 3% to 14% of the full-length protein value (Table 2).

The pH-activity profiles for the different constructs are shown in Figure 2. Both the full-length protein and the one with the 90 N-terminal residues removed (D1,2,3_{91–1241}) exhibited a type A pH-activity profile (Figure 2A). Peptides encompassing complete individual domains (D1_{92–486}, D2_{489–862}, and D3_{791–1241}) also exhibited type A pH-activity profiles (Figure 2B–D). In contrast, the removal of about 50 N-terminal residues in peptides of each individual domain resulted in type B activity. At pH 8, peptides D1_{167–486}, D2_{535–897}, and D3_{927–1241}, lacking the first 55, 47, and 62 amino acids of their N-termini (Figure 1), retained about 40%, 50%, and 70% of the pH 6.3 luciferase activity, respectively (Figure 2B–D). The amino acid sequences for the N-terminal regions of the three domains are compared in Figure 3; the first amino acid of each of the truncated peptides is marked with an asterisk at its upper left corner.

Histidines Are Responsible for Loss of Activity at Alkaline pH. Four intramolecularly conserved histidine residues are present among the first 80 amino acids in the N-terminal region of the three domains (Figure 3), and the *pK* of the imidazolium of histidine is between 6.5 and 7.0 (12), close to the *pK* of ~6.8 for the loss of luciferase activity in the alkaline region (13; Figure 2). Peptides in which any one of the four conserved histidines in domain 2 was replaced by alanine retained good activity at pH 6.3 (Table 3), while at all sites such individual substitutions resulted in greatly increased activities at pH 8 (Figure 4). Peptides with multiple substitutions also retained good specific activities at pH 6.3 (Table 3) but large increases of activity at pH 8, such that activities at pH 8 and 6.3 are about equal with two or more sites replaced by alanine. The results suggest that the histidines in their uncharged forms strongly inhibit the reaction in an apparently novel way; a mechanism is not immediately evident.

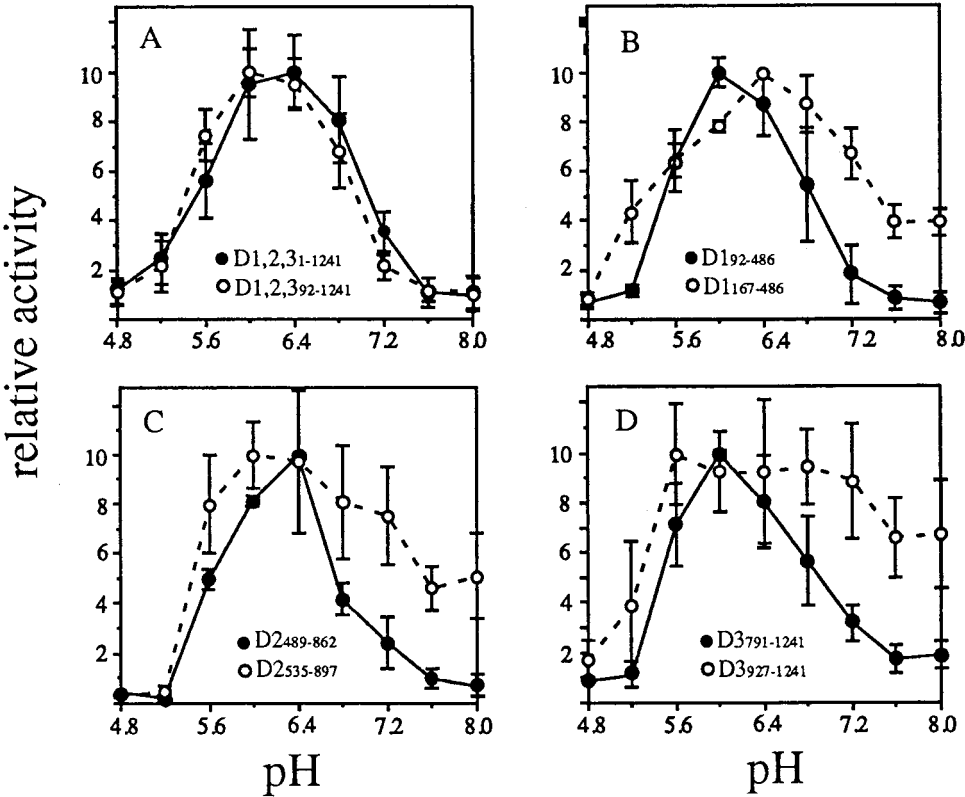


FIGURE 2: pH-Activity profiles for fusion proteins, showing that the absence of amino acids from the N-terminal portions of individual domains results in a greater luciferase activity in the alkaline region relative to that at the optimum. All curves are plotted normalized to the peak activities. (A) Full-length luciferase D1,2,31-1241 compared to D1,2,392-1241 having all three domains but lacking the N-terminus. (B) Domain 1 peptide D192-486 compared to the truncated D1167-486 (H1 and H2 removed). (C) Domain 2 peptide D2489-862 compared to the truncated D2535-897 (H1 and H2 removed). (D) Domain 3 peptide D3791-1241 compared to the truncated D3927-1241 (H1, H2, and H3 removed).

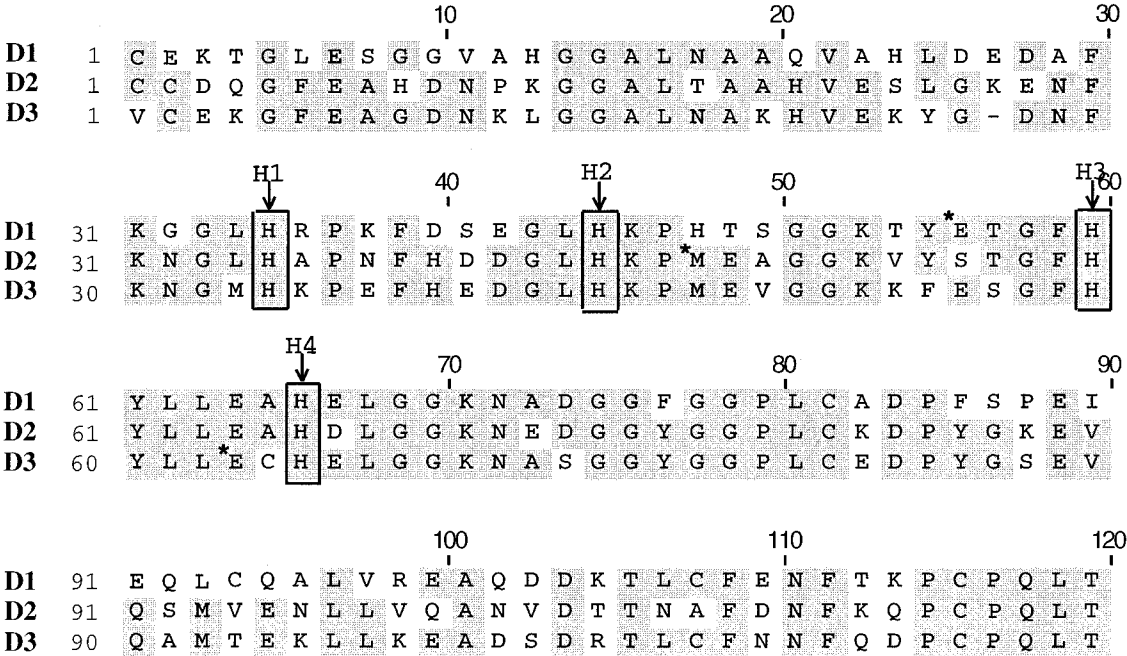


FIGURE 3: Peptide sequence alignment of the first 120 N-terminal amino acids of the three intramolecularly homologous domains, with the consensus residues shaded. The first residue of each repeated domain is numbered 1; note that D3 lacks the residue corresponding to 27 in D1 and D2. The first amino acid residues in the truncated domains are 56, 48, and 63, marked with asterisks at their upper left corners. Residues 1-29 have 28% sequence identity for all three domains; residues 30-82, 64% identity; residues 83-114, 31% identity; and (not shown) residues 115-281, 95% identity (5).

As noted above, the central regions (residues 115-281) are highly conserved, being ~95% identical pairwise for all three domains. At the same time, the region in which the

conserved histidines are located (residues 30-82) is more highly conserved (64% identical) than sequences before and after (residues 1-29, 28% identical; residues 83-114, 31%

Table 3: Luciferase Specific Activities at pH 6.3 of Domain 2 (D2₄₈₉₋₈₆₂) GST Fusion Peptides with Histidines as Indicated Replaced by Alanines^a

peptide	specific activity
D2 ₄₈₉₋₈₆₂	100 ± 4.8
H1	75 ± 7.5
H2	116 ± 20.1
H3	89 ± 18.2
H4	51 ± 7.6
H1,2	75 ± 15.7
H1,2,3	47 ± 8.1
H1,2,3,4	24 ± 3.8

^a Expression, purification, and measurements carried out as described in Table 2. Peptides were named as in the caption to Figure 4 (see Figure 3). One activity unit corresponded to 2.5×10^{11} quanta s⁻¹ μmol⁻¹.

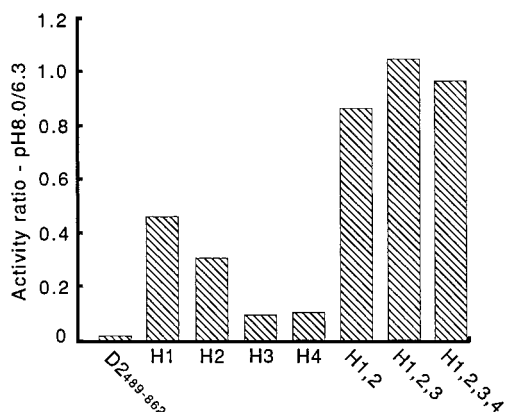


FIGURE 4: Bar graph showing the effects of substitutions of histidines for alanines on the ratio of luciferase activity at pH 8 to that at pH 6.3 in domain 2 peptides. Values represent the ratios of the averages of six or more individual activity measurements. H1, H35A; H2, H45A; H3, H60A; H4, H66A; H1,2, H35,45A; H1,2,3, H35,45,60A; H1,2,3,4, H35,45,60,66A.

identical). This is indicative of a functional importance for the region between residues 30 and 82, which is concluded to be the regulation of activity by pH.

Residues Responsible for pH Profiles Are Not Essential for Activity. The typical bell-shaped dependence of enzyme activity on pH is commonly attributed to the ionization of acidic and basic groups of key amino acids that are important in the catalytic reaction mechanism. In such cases there is a requirement for a particular ionic form of each of two such amino acids, with pK values to the acidic and basic sides of the pH optimum defining the shape of the curve (12).

A change in the tertiary structure of the enzyme can also be responsible for a pH-dependent change in activity without affecting the catalytic rate constant. In this case, changes in the ionization state of an amino acid that is not directly involved in the catalytic mechanism may be responsible. In chymotrypsin, for example, the loss of activity on the basic side is attributed to the removal of the positive charge from the amino-terminal isoleucine so that its interaction with a negatively charged aspartate is lost. This alters the protein conformation so that access to the active site is blocked, as measured by a decreased affinity for substrate (14). The inactivation of this isoleucine residue also results in an inactive enzyme, since the availability of the active site is dependent upon the interaction of the charged isoleucine with aspartate.

The pH regulation of *Gonyaulax* luciferases is different, possibly unique. For both full-length and single domains the low activity in the alkaline region cannot be attributed to the titration of a residue that is required for activity, since the activity of the enzyme molecule with histidines changed to alanines is not greatly changed at pH 6.3 and, indeed, exhibits an activity at pH 8 that may be equal to that at pH 6.3 (Figure 4).

A possible explanation for the lower activity at pH 8 in peptides having the native sequence is that the residue(s) titrated in the alkaline region act to inhibit activity by causing a conformational change in the protein, but whether this is by somehow altering the catalytic rate constant or by restricting substrate accessibility cannot be determined from the data. If histidine is indeed the amino acid involved, as the data suggest, the uncharged form is responsible for the loss of activity at pH 8, and surprisingly, its action is not duplicated by a different uncharged amino acid, alanine. The results further indicate that the same behavior occurs in both the full-length and the isolated complete single-domain molecules, meaning that the putative conformation changes would occur in three different parts of the native luciferase.

It is well-established that pK values for ionizable amino acid side chains in protein can be highly dependent upon the microenvironment and may differ by orders of magnitude from those measured in water (15, 16). Thus, an alternative possibility to consider is that the pKs of the histidines in question are shifted to the alkaline region so that they remain charged at pH 8, able to interact with negatively charged acidic amino acids such as glutamate or aspartate, whose pKs might be likewise shifted; for example, to pH 6.7. Such an interaction in the alkaline region could be responsible for the loss of activity, for example, by blocking access to the active site. There are conserved glutamates at positions 7 and 64 and conserved Glu/Asp at positions 42 and 67. However, activity continues to remain low at pH 9 in the native sequence peptide where the histidines might be expected to have lost charge, while the activity is still high with histidines replaced by alanine.

Protease Action and Regulation of Flashing in Vivo. Implications of these results for our understanding of the action of proteases and the differences between the pH profiles of the two molecular weight fractions reported earlier are now evident. The results are consistent with a model in which proteases preferentially attack bonds in the N-terminal regions of the three domains, thereby releasing peptides with the catalytic domain intact but the inhibitory N-termini removed. This suggests a structure for the full-length luciferase with the central catalytic regions of the three domains being tightly folded and less accessible to protease attack than the intervening sequences. At the same time, the putative spacer regions would play a crucial role in modulating the activity of each catalytic center through pH-dependent conformational changes.

In the living cell, the importance of pH changes on the luciferase activity relates to the origin and regulation of the flashes of bioluminescence. These flashes originate from numerous (~400 per cell) small (0.4 μm in diameter) organelles called scintillons (17), enveloped by the vacuolar membrane, where the luciferase, luciferin, and luciferin-binding protein are localized (6, 8, 18). The postulate that the flash is caused by a rapid and transient pH change in the

scintillon is supported by the fact that native luciferase is maximally active at pH 6.3 and inactive at pH 7.5 and above; the luciferin substrate is sequestered by its binding protein above pH 7.5 but not at pH 6 (10); the pK is at 6.8 for both proteins (13). A vacuolar membrane action potential (9) is postulated to result in a brief opening of channels, allowing protons to enter the scintillon, thus causing a rapid and transient pH change and a flash. The inhibition of activity at pH 8 may also have functional importance in preventing luminescence in the cytoplasm, where luciferase and binding protein occur in association prior to their incorporation in the scintillon.

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